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Plant Expression of a Bacterial Cytochrome P450 That Catalyzes Activation of a Sulfonylurea Pro-Herbicide

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The *Streptomyces griseolus* gene encoding herbicide-metabolizing cytochrome $P450_{SU1}$ (CYP105A1) was expressed in transgenic tobacco (*Nicotiana tabacum*). Because this P450 can be reduced by plant chloroplast ferredoxin in vitro, chloroplast-targeted and nontargeted expression were compared. Whereas $P450_{SU1}$ antigen was found in the transgenic plants regardless of the targeting, only those with chloroplast-directed enzyme performed $P450_{SU1}$-mediated N-dealkylation of the sulfonylurea 2-methylethyl-2,3-dihydro-*N*-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benzoisothiazole-7-sulfonamide-1,1-dioxide (R7402). Chloroplast targeting appears to be essential for the bacterial P450 to function in the plant. Because the R7402 metabolite has greater phytotoxicity than R7402 itself, plants bearing active $P450_{SU1}$ are susceptible to injury from R7402 treatment that is harmless to plants without $P450_{SU1}$. Thus, $P450_{SU1}$ expression and R7402 treatment can be used as a negative selection system in plants. Furthermore, expression of $P450_{SU1}$ from a tissue-specific promoter can sequester production of the phytotoxic R7402 metabolite to a single plant tissue. In tobacco expressing $P450_{SU1}$ from a tapetum-specific promoter, treatment of immature flower buds with R7402 caused dramatically lowered pollen viability. Such treatment could be the basis for a chemical hybridizing agent.

P450 monooxygenases play a central role in plant response to a variety of environmental challenges, including herbicide and other pesticide treatments (Cole, 1983; Durst, 1991). The best examples of this role are those P450s that can chemically alter an herbicide to a form with reduced phytotoxicity, resulting in much higher herbicide tolerance in the plant where this metabolism occurs (Frear et al., 1969, 1991; Sweetser et al., 1982; Jacobson and Shimabukuru, 1984; Brown, 1990). Significant variability in P450 enzyme activity exists among plant species, both in the presence of distinct enzymic forms and in their range of substrate specificity. This variability is a major determinant in herbicide selectivity, the differential herbicide sensitivity of weed and crop species. Altering herbicide selectivity, particularly conferring resistance in a crop plant, might be accomplished by expression of the appropriate P450 enzyme in a sensitive plant. Although a few plant P450 proteins have been purified and a growing number of genes have been cloned (Nelson et al., 1993), none of these are enzymes primarily responsible for herbicide metabolism.

Streptomyces griseolus, a soil bacterium, has two inducible P450 monooxygenase systems capable of metabolizing a variety of sulfonylurea herbicides (O'Keefe et al., 1988). The metabolites produced by these enzymes are in many cases identical to those made by analogous plant enzymes, and because of this they have served as a model system for plant P450 herbicide metabolism (O'Keefe et al., 1987; Harder et al., 1991). The *S. griseolus* enzyme systems are dependent on the two terminal oxidase proteins, Cyts $P450_{SU1}$ and $P450_{SU2}$ (systematically designated CYP105A1 and CYP105B1), for both oxygen redox chemistry and substrate specificity. The accessory proteins involved in delivery of the reducing equivalents essential for monooxygenase chemistry consist of co-induced Fds and a poorly defined NAD(P)H oxidoreductase operating in the electron transfer sequence NAD(P)H, oxidoreductase, Fd, and P450. The genes for both P450s and both Fds have been cloned and sequenced and can be actively expressed in other *Streptomyces* species (Omer et al., 1990; O'Keefe et al., 1991).

Previously, it was reported that other Fds could substitute for the endogenous Fds in vitro, and indeed $P450_{SU1}$ monooxygenase functions with >50% efficiency using chloroplast Fd from higher plants (O'Keefe et al., 1991, 1993). This Fd comes from the stromal space of the chloroplast, where it functions in the transfer of reducing equivalents between PSI and Fd:NADP oxidoreductase. The ability to interact with Fd(s) that are already present in plant tissues suggests that it might be possible to transform plants with the *S. griseolus* P450 genes and obtain functional expression by assuring that the P450 protein is localized in the stromal space of the chloroplast. Nuclear-encoded chloroplast-targeted proteins, such as the light-harvesting Chl *a/b* protein or the small subunit of Rubisco, are normally imported into the chloroplast following cytoplasmic translation (Dunsmuir, 1985;

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Abbreviations: ALS, acetolactate synthase; chlorimuron ethyl, *N*-[(4-chloro-6-methoxy-pyrimidin-2-yl)aminocarbonyl]-2-ethoxycarbonylbenzenesulfonamide; I_{50}, inhibitor concentration resulting in 50% inhibition of activity; P450, cytochrome P450; R7402, 2-methylethyl-2,3-dihydro-*N*-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benzoisothiazole-7-sulfonamide-1,1-dioxide.

Dean et al., 1989). An amino-terminal transit sequence on these proteins directs the translocation of the nascent polypeptide across the chloroplast envelope. The transit sequence is removed to produce the mature protein. Attaching such a sequence to the amino terminus of the P450 and placing the resulting coding region under the control of a plant promoter should result in a plant gene that produces a chloroplast-localized P450.

In this report we describe the expression of *S. griseolus* $P450_{SU1}$ in tobacco (*Nicotiana tabacum*). Targeting the mature P450 to the chloroplast results not only in detectable expression of the protein but in appearance of a measurable phenotype as well. This phenotype is based on the $P450_{SU1}$-mediated metabolism of the sulfonylurea R7402 from its normally nonherbicidal form to one that is highly phytotoxic. The ability to potentiate phytotoxic effects suggests that this plant-expressed P450 may have other practical uses. R7402 can be used in a negative selection screen to identify transformants that are expressing the $P450_{SU1}$ gene. Negative selection screens are useful, for example, in experiments involving homologous recombination (Capecchi, 1989) or in those designed to identify *trans*-acting factors involved in regulation of gene expression (Karlin-Neumann et al., 1991). Expression of $P450_{SU1}$ from a tissue-specific promoter allows the phytotoxic effect to be potentiated in only one tissue type. In these plants $P450_{SU1}$ is expressed in the tapetum cells, which surround and provide nutrients to the developing pollen grains. In the absence of R7402, pollen develops normally; however, treatment with R7402 results in production of nonviable pollen. This effect may have some use in producing a chemical male-sterilizing agent to be used for hybrid seed production. A preliminary report of some of the findings presented here has appeared previously (O'Keefe et al., 1992).

MATERIALS AND METHODS

Nontargeted P450 Gene Construction

A plasmid containing the 2.4-kb *Bam*H1 fragment from *Streptomyces griseolus*, which includes the entire coding region for $P450_{SU1}$ (Omer et al., 1990), was subjected to exonuclease III deletion leaving an end point 6 bp 3′ to the termination codon of $P450_{SU1}$, followed by a *Hin*dIII site. This plasmid was cut with *Hin*dIII, blunted, and cut at an *Eco*RI site located upstream of the translation-initiating ATG. The *Eco*RI-blunt fragment was cloned into *Eco*RI and *Hin*cII-digested pUC118. The $P450_{SU1}$ coding region could then be excised as a 1.3-kb *Eco*RI-*Pst*I fragment. The polyadenylation signal sequence region from a petunia Rubisco small subunit gene was available in a plasmid, pSSU3303 (Dean et al., 1989), with a *Bgl*II site at the translational stop. A 1.45-kb blunt-*Bam*HI fragment derived from the *Bgl*II-*Bam*HI fragment containing the polyadenylation signal region was subcloned into pUC118, making it available as a *Pst*I-*Bam*HI fragment. A three-component ligation was performed with the $P450_{SU1}$-containing *Eco*RI-*Pst*I fragment, the polyadenylation signal *Pst*I-*Bam*HI fragment, and *Bam*HI-*Eco*RI-cleaved pUC118. The resulting plasmid was subjected to in vitro mutagenesis (Kunkel, 1985) to introduce a *Sca*I site at the translation start codon of $P450_{SU1}$, creating the plasmid pSU16. The 35S promoter of cauliflower mosaic virus and the 5′ untranslated region from a petunia Chl *a/b*-binding protein gene (Cab22L) were obtained from the plasmid p35S(J):Cab22L-CH (Harpster et al., 1988) as a 1.2-kb *Eco*RI-*Nco*I (blunted) fragment and ligated to the *Eco*RI and *Sca*I sites in pSU16, giving pSU17. When the blunted *Nco*I and *Sca*I sites are ligated, the ATG start of the $P450_{SU1}$ coding region is restored. pSU17 thus contains a 35S promoter, a 5′ untranslated region from Cab22L, a $P450_{SU1}$ coding region, and a Rubisco small subunit polyadenylation signal.

Rubisco Small Subunit Promoter, Chloroplast Targeting

A 1.8-kb *Cla*I (blunted)-*Bam*HI fragment containing an intronless Rubisco small subunit gene was obtained from pSSU3019 (Dean et al., 1989) and cloned into *Sma*I-*Bam*HI-cleaved pUC8 to add an *Eco*RI site onto the 5′ end of the promoter region. Site-directed mutagenesis was used to introduce an *Eco*RV site after the codon for amino acid 12 of the mature Rubisco sequence. An *Eco*RI-*Eco*RV fragment from this plasmid was cloned into *Eco*RI-*Sca*I-cleaved pSU16, creating pSSU-SU11, a plasmid containing the Rubisco promoter, the 5′ untranslated region, the coding region for the transit peptide plus 12 amino acids of the mature protein, the $P450_{SU1}$ coding region, and the Rubisco polyadenylation signal. Site-directed mutagenesis was used to delete the coding region for the amino acids from mature Rubisco, leaving a perfect fusion of the transit sequence with $P450_{SU1}$ in the plasmid pSSU-SU12.

Chl *a/b*-Binding Protein Promoter, Chloroplast Targeting

A 950-bp *Bal*I-*Sac*I fragment of the petunia Cab22L gene (Dunsmuir, 1985), containing the Cab22L promoter, the transit peptide coding sequence, and part of the mature protein coding sequence, was cloned into *Sma*I-*Sac*I-cleaved pBluescript KS$^+$, creating pCab22LT. A *Sca*I site was created by site-directed mutagenesis after the codon for amino acid 14 in the mature Chl *a/b* protein and a 1.2-kb *Eco*RI-*Sca*I fragment from the resulting plasmid was subcloned into *Eco*RI-*Sca*I-cleaved pSU16, creating pCab-SU11. To investigate if more of the mature Chl *a/b* protein coding region would give better uptake of the P450 into the chloroplast, an oligonucleotide was made to loop in 39 nucleotides that code for amino acids 15 to 27 of the mature protein between amino acid 14 and the start of the $P450_{SU1}$ coding region, creating pCab-SU13. A third Chl *a/b* promoter-transit sequence construction was made from pCab22LT by inserting a *Sma*I site after the codon for amino acid 19 of the mature Chl *a/b* protein. A 1.2-kb *Eco*RI-*Sma*I fragment from the resulting plasmid was subcloned into *Eco*RI-*Sca*I-cleaved pSU16, creating pCabSU12.

Anther-Specific Expression Vectors

The promoter of the tobacco TA29 gene (Goldberg, 1988) was used to provide tapetum-specific gene expression. An *Nco*I site was added at the translation-initiating ATG of a 1.5-kb TA29 promoter fragment by site-directed mutagenesis.

A *Sca*I-*Bam*HI fragment from pSU17, described above, containing the $P450_{SU1}$ coding region and Rubisco small subunit polyadenylation signal, was ligated to the blunted *Nco*I site at the 3′ end of the TA29 promoter and a following *Bam*HI polylinker site, creating plasmid pTZA-SU1. In a second construction a chloroplast transit signal sequence was added. The *Nco*I site at the 3′ end of the TA29 promoter was ligated to the *Nco*I site located within the SU1 coding region. Then an *Nco*I-*Nco*I fragment from pSSU-SU11, containing the coding region for the Rubisco small subunit transit sequence plus 12 amino acids of mature protein and the 5′ coding sequence of $P450_{SU1}$, was added at the *Nco*I junction site. A clone with the correct orientation of the insert was identified by restriction digests and named pTZA-T-SU1.

Binary Vector and Tobacco Transformation

The wide host range vector pAGS135 (Dean et al., 1988) is derived from pAGS112 (van den Elzen et al., 1985) by removal of the *Xho*I site outside the T-DNA right border; pAGS112-transformed plants were used as controls in many experiments. The six constructs, pSU17, pSSU-SU11, pSSU-SU12, pCab-SU11, pCab-SU12, and pCab-SU13, were digested with *Bam*HI and inserted into pAGS135 at its unique *Bam*HI site, creating the binary vectors pSU18, pSSU-SU111, pSSU-SU121, pCab-SU111, pCab-SU121, and pCab-SU131. These plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) using triparental matings. The resulting strains were then co-cultivated with protoplasts or leaf discs of *Nicotiana tabacum* cv Wisconsin 38 (Horsch et al., 1985; van den Elzen et al., 1985) and kanamycin-resistant transformants were selected. Tobacco transformants in this study are named according to the transformation vector without the plasmid designation, with a decimal number to indicate an individual transformant (e.g. SSU-SU121.3).

For the anther-specific vectors, the A-SU1 and A-T-SU1 chimeric genes were each added as an *Asp*718-*Bam*HI fragment to the binary vector pZS96. This vector contains the origin of replication and ampicillin-resistance gene from pBR322, the replication and stability regions of the *Pseudomonas aeruginosa* plasmid pVS1 (Itoh et al., 1984), T-DNA borders described by van den Elzen et al. (1985), and a plant kanamycin-resistance selection marker. The resulting plasmids were transferred into *A. tumefaciens* strain LBA4404 by direct DNA uptake (An et al., 1988) and the resulting agrobacteria were used to generate transformed tobacco plants as described previously (Odell et al., 1990) using a kanamycin selection.

Analysis of Sulfonylurea Metabolites

First-generation progeny of the primary tobacco transformants and suitable controls were grown in a growth chamber and leaves were removed from the main stalk to promote lateral shoot growth. Leaves from these shoots were of more uniform size for metabolism measurements. Total leaf protein from the transformants was analyzed on western blots to verify that they were expressing $P450_{SU1}$. Leaves were excised under water with a scalpel and transferred to cups with the uptake solution (20 μg/mL sulfonylurea in 1 mM K phosphate, pH 7.0) and allowed to take up the solution at 22°C in the light (200 $\mu E\ s^{-1}\ m^{-2}$). After the uptake period, the leaves were transferred to cups containing only phosphate buffer for the remaining time. The leaves were then homogenized in 80% acetone and centrifuged to remove tissue debris, and the acetone from the supernatant was removed by evaporation. The resulting aqueous extracts were acidified to pH 2 to 3 with H_2SO_4 and extracted three times with methylene chloride. The extracts were analyzed by HPLC as previously described (Sweetser et al., 1982). Because there was substantial leaf-to-leaf variation in the amount of uptake and metabolism, even among leaves from a single plant, the results of several single-leaf determinations were averaged.

Herbicide Spray Treatment

Plants were grown from seed in a commercial potting soil for 47 d, at which time the plants had four to five fully expanded leaves. One leaf was removed for P450 antigen analysis prior to application of a spray treatment equivalent to 1 g/ha R7402 in 90.2% acetone, 4.8% glycerol, 4.8% water, and 0.24% Tween-20 (AGWT). The plants were returned to the greenhouse for 22 d before further examination.

Negative Selection of Transformed Tobacco Seed

Growth medium was prepared from Murashige minimal organics medium (Gibco), 8 g/L agar, 50 ng/mL biotin, 0.5 μg/mL folic acid, 2 μg/mL Gly, and 100 μg/mL *myo*-inosotol. Either 50 nM R7402 or 200 μg/mL kanamycin sulfate was present in some experiments. Tobacco seed was surface sterilized for less than 30 min with 30% chlorine bleach, 0.1% SDS prior to placement on the medium and grown under illumination (100 $\mu E\ s^{-1}\ m^{-2}$) at 22°.

Herbicide Application and Pollen Analysis (Anther-Specific Promoters)

Transformants containing the $P450_{SU1}$ gene expressed from the TA29 promoter were grown to maturity. During flowering, each plant was sprayed with 14 mL of varying concentrations of an R7402 solution in AGWT using a hand-held atomizer. The total amount of R7402 applied to each plant ranged between 74 μg and 2 mg. Buds and flowers further along in development than stage 3, as described by Koltunow et al. (1990), were removed prior to spraying. Stage 3 is identified by the emergence of the corolla from the calyx and is the point when pollen grains begin to form. Anthers are green at this stage. At time points between 1 and 3 weeks after spraying, pollen was collected into germination medium (Harris et al., 1989) and incubated overnight. Germination and growth of pollen tubes were analyzed under a light microscope.

General Techniques

Protein was quantitated by the Bio-Rad protein assay, and P450 antigen was quantitated using polyclonal $P450_{SU1}$ antisera (O'Keefe et al., 1987) using western blots or immunoslot blots. Tobacco leaf mRNA was extracted as previously

described and analyzed either by primer extension or northern blot analysis (Dean et al., 1985). A small-scale procedure was used to prepare total RNA from anthers (Verwoerd et al., 1989).

RESULTS

Expression and Function of $P450_{SU1}$ in Tobacco

Our initial goal was to use bacterial $P450_{SU1}$ to modify the herbicide sensitivity of tobacco. In vitro experiments with purified proteins demonstrated that $P450_{SU1}$ could use chloroplast Fd as an immediate reductant (O'Keefe et al., 1991, 1993), suggesting that this P450 could be made to function in a plant provided that it was directed to the chloroplast stromal space. Nonetheless, it was not clear whether nontargeted cytoplasmic expression, which is significantly simpler, could also result in functional expression of $P450_{SU1}$. Several chimeric genes were constructed (Fig. 1) to test expression from different promoters and to determine whether different chloroplast transit sequences added onto the amino terminus of $P450_{SU1}$ could direct chloroplast uptake and affect activity of the mature protein. Of these, SU18 used the 35S promoter of cauliflower mosaic virus and was intended to result in cytoplasmic accumulation of the P450. Only one of the chloroplast-targeting constructions, SSU-SU121, resulted in a "perfect" fusion of the transit sequence and $P450_{SU1}$. Because of reports that retention of some part of the amino terminus from the mature protein from which the transit sequence is derived leads to better chloroplast uptake (Kavanagh et al., 1988), the remainder of the constructions retained up to 27 of these "extra" amino acids.

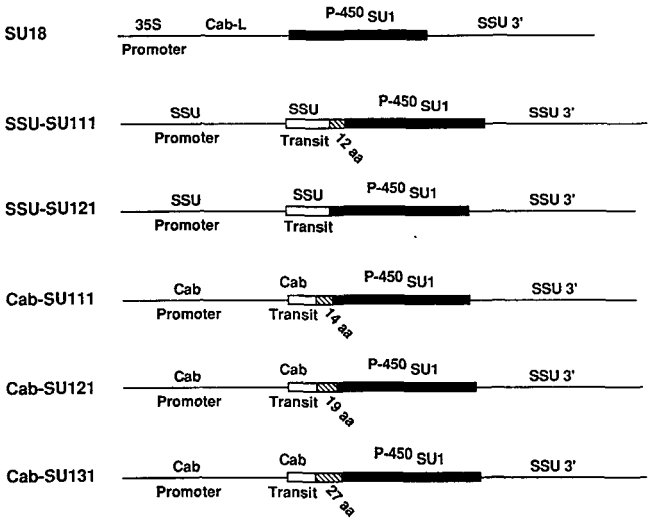

Figure 1. The DNA constructions for tobacco $P450_{SU1}$ expression. The origin and assembly of these chimeric genes are described in detail in "Materials and Methods." The components were obtained from the *S. griseolus suaC* gene encoding $P450_{SU1}$, the cauliflower mosaic virus 35S promoter, the petunia SSU301 gene encoding Rubisco small subunit, and the petunia Cab22L gene encoding Chl *a/b*-binding protein. The black box represents the $P450_{SU1}$ coding region, the open box is a chloroplast transit sequence from either the SSU301 gene (SSU) or the Cab22L gene (Cab), and the hatched box is the coding region for some amino acids from the amino terminus of the mature protein from which the transit sequence is derived. SSU 3', 3' untranslated region including the polyadenylation signal sequence from the SSU301 gene. Cab-L, 5' untranslated leader sequence from the Cab22L gene. Maps are not to scale.

Following transformation with *A. tumefaciens* harboring the chimeric $P450_{SU1}$ genes, transformants were selected by kanamycin resistance and differentiated plants were rooted and allowed to mature. These plants were screened using primer extension analysis of $P450_{SU1}$ mRNA to identify those expressing the $P450_{SU1}$ gene (data not shown). By this criterion, 17 of the 21 kanamycin-resistant individuals transformed with pSU18, and about 90% of the 50 kanamycin-resistant individuals transformed with the five genes that included chloroplast transit signals, were expressing detectable levels of $P450_{SU1}$. Individuals with the highest levels of $P450_{SU1}$ mRNA were identified and allowed to self-pollinate. Plants grown from these segregating populations of seed were used exclusively for further characterization.

As Figure 2 shows, plants exhibited a variety of expression levels of $P450_{SU1}$ antigen. Nonchloroplast-targeted expression (SU18.14) was within the same range of expression observed with the chloroplast-targeted P450. In plants containing the chloroplast-targeting constructions, no immature P450 containing either the 3.5-kD Cab or the 6-kD Rubisco transit peptide was apparent. Some smearing of the $P450_{SU1}$ band is evident in samples of Cab-SU131.5 and SSU-SU111.5. Since the samples in Figure 2 include both chloroplast and cytoplasm contents, this smearing suggests that there may be incomplete chloroplast uptake or inaccurate cleavage of the transit peptide inside the chloroplast of some transformants. All of the SSU-SU121.3 plants tested exhibited distinct 45-kD $P450_{SU1}$ bands on western blots, and in a separate experiment (not shown), the antigen was found in isolated chloroplast stromal contents from SSU-SU121.3 leaves. These results demonstrate that there is successful expression of the $P450_{SU1}$ polypeptide in tobacco, and suggest that it can be properly targeted to the chloroplast stromal space with correct proteolytic cleavage of the transit peptide.

The results above do not provide any information on whether the bacterial P450 is able to fold properly, have heme inserted, and interact successfully with Fd in the chloroplast. We chose to use the activity of the enzyme to address these issues. One of the best substrates for $P450_{SU1}$ is the sulfonylurea compound R7402 (O'Keefe et al., 1991, 1993). This compound is a very poor inhibitor of ALS, the target enzyme of the sulfonylurea herbicides (Chaleff and Mauvais, 1984). The N-dealkylation of R7402 catalyzed by $P450_{SU1}$ (see Fig. 3) produces a metabolite that is nearly 500-fold more inhibitory to ALS than R7402. The increased ALS inhibition of the R7402 metabolite should be manifest as increased phytotoxicity and is the basis for calling R7402 a "proherbicide." Formation of the N-dealkylated metabolite by control and transgenic tobacco leaves is shown in Figure 4. The metabolite was observed only in plants with a chloroplast-targeted P450; levels of metabolite in the control plants and in those with cytoplasmic-expressed P450 were below the limit of detection. The amount of metabolite formed in 5 h in the chloroplast-P450 leaves corresponds to about 20% of the total R7402 taken up in Cab-SU131.5, and for this

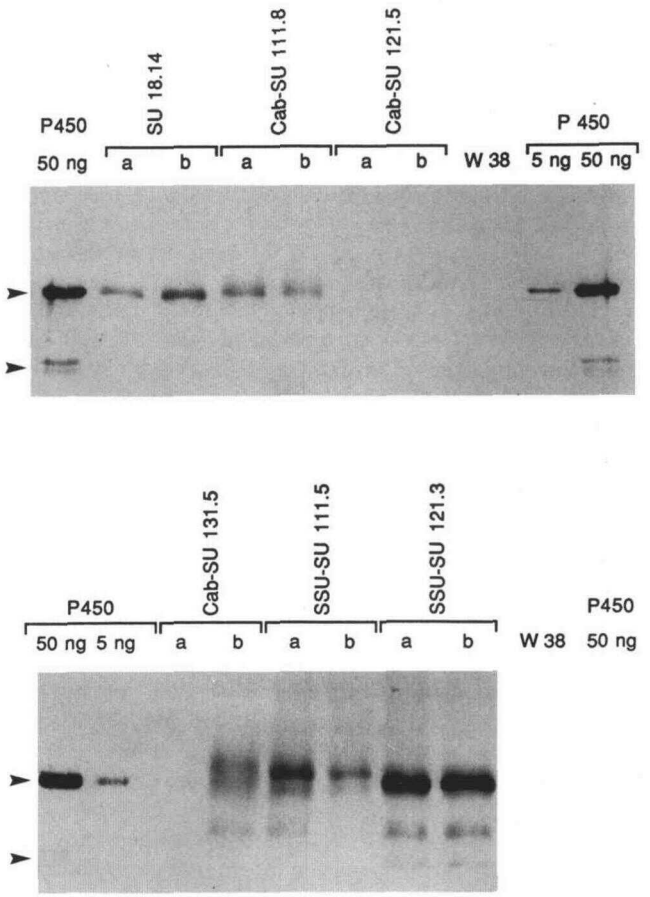

Figure 2. Western blot analysis of total leaf protein from transgenic tobacco. Twenty micrograms of total leaf soluble protein were loaded in the lanes indicated. Tissue from two individual plants (a or b) of a segregating population of seed was analyzed. The lanes labeled "P450" contain the indicated quantities of $P450_{SU1}$, purified as described (O'Keefe et al., 1988). W38, Wisconsin 38 control. In both panels the upper and lower arrowheads indicate molecular masses of 45 and 39 kD, respectively.

plant we calculate the rate of metabolism at 0.3 μg g^{-1} leaf h^{-1}.

In contrast to the readily measurable metabolism of R7402, the metabolism of chlorimuron ethyl, a compound that is detoxified by the action of $P450_{SU1}$, was predictably much less. From in vitro studies, the rates of formation of two metabolites from chlorimuron ethyl are each expected to be 335 times slower than R7402 dealkylation (O'Keefe et al., 1993). Significant background metabolism of chlorimuron ethyl in control tobacco made the analysis of any enhanced metabolism in the transformants difficult. Measurement of metabolism over a 21-h period showed that the disappearance of chlorimuron ethyl and the appearance of its two metabolites were approximately 2 times faster in SSU-SU111.5, and 1.5 times faster in Cab-SU131.5, than in the control AGS112 plant.

Phenotypically, all of the transformed tobacco plants were indistinguishable from control plants from germination to flowering. The results of spray treatment of these plants with

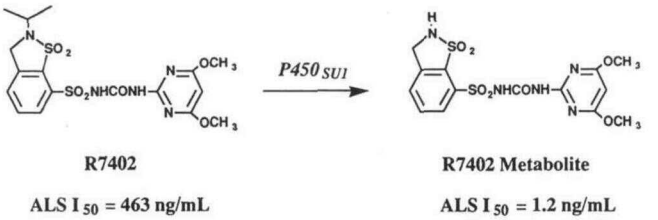

Figure 3. N-Dealkylation of the sulfonylurea R7402 by $P450_{SU1}$. The I_{50} values are determined with a crude preparation of wild mustard ALS.

R7402 are shown in Figure 5. It is clear from this figure that the growth of plants with the chloroplast-directed P450 was arrested at the time of R7402 treatment 22 d earlier, and serious injury is evident. In contrast, the control plants grew vigorously after treatment. Only marginal growth inhibition occurred in the plants with nonchloroplast-targeted P450, even though these had substantial levels of $P450_{SU1}$ antigen (Fig. 2). Similar results were obtained at higher application rates of R7402 (4 g/ha), but at 16 g/ha there was significant damage to the control plants as well. The sensitivity of the transformants confirms that the phytotoxic metabolite of R7402 is being produced in plants expressing $P450_{SU1}$; maximal activity is obtained when the protein is targeted to the chloroplast.

Plant Negative Selection

The experiments shown in Figures 4 and 5 were performed on plants that were first-generation progeny (selfed) of the

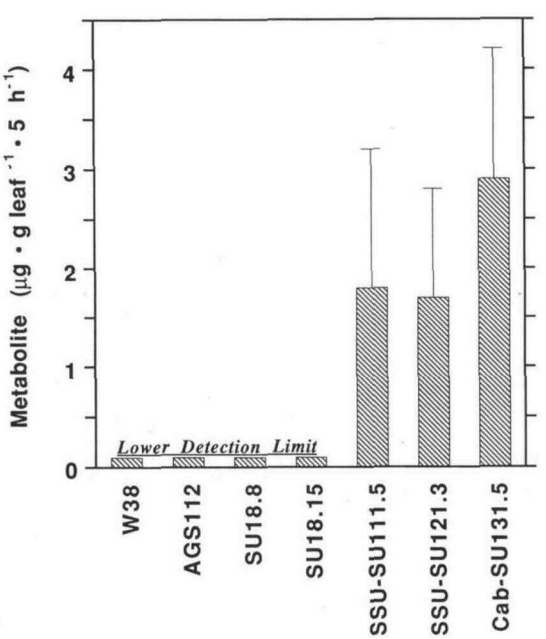

Figure 4. R7402 metabolite formation by excised tobacco leaves. Metabolite was extracted from leaves of the indicated plants after 2 h of uptake and 5 h of incubation. Values shown are the average of separate determinations on four to eight leaves from at least two individual plants, with the exception of SU18.15 (two leaves from a single plant). Error bars indicate SD.

primary transformants. In this generation the transgenes would be segregating with frequencies that depend on the number of insertion loci in the primary transformants. To account for this segregation, the experiments described in these figures were performed on individuals that were prescreened and shown to contain $P450_{SU1}$ antigen. Although very reliable, this is a cumbersome means to test for expression, and other means were subsequently used to test for the number of transgene loci and/or for P450 expression. Segregation of the kanamycin-resistance selection marker was assayed by germination of seed on kanamycin-containing medium. By this criterion all of the transformants discussed thus far were derived from primary transformants with more than two transgene loci, with the exception of SU18.14 and Cab-SU121.5, which were derived from a parent with two loci, and the control AGS112, which had a single kanamycin-resistance locus. As Figure 5 shows, R7402 sensitivity is an even better screening tool, since this tests for function of the P450 itself. Figure 6 compares a screening of control seed and seed from a transformant with two kanamycin-resistance loci on R7402-containing medium. Only the individual that has no $P450_{SU1}$ gene, which appears at the expected frequency of 1 in 16 plants, grows in this medium. Similar results, but with lower frequencies of healthy seedlings, were obtained with the other chloroplast-directed transformants (SSU-SU111, SSU-SU121, Cab-SU111, Cab-SU131), confirming the kanamycin marker results that fewer than two transgene loci are present. Individuals that are negatively selected by this means (i.e. those that are inhibited) are easily rescued from this treatment. Because they have expanded cotyledons but no developed roots, they can be removed and placed on fresh medium without R7402, where growth resumes after a lag of several weeks.

Selective Destruction of Plant Tissue

The ability of the compound R7402 to potentiate lethal effects on plant tissue only if $P450_{SU1}$ is present suggests a possible technique for selective tissue destruction. This would be possible if the expression of the P450 were placed under the control of a tissue-specific promoter. The protein would be expressed specifically in that tissue, and the damaging effects of metabolism would take place in the tissue only after treatment with R7402. Provided that the toxic R7402 metabolite did not diffuse to neighboring tissue, the effects would be confined to a single tissue type. Expression of the tobacco TA29 promoter is specific to the tapetum cells in the anther of the flower (Koltunow et al., 1990). These cells provide nutrients to the developing pollen, and disruption of this function by P450-mediated production of the toxic R7402 metabolite should result in damage to the pollen. Figure 7A shows chimeric $P450_{SU1}$ genes under the control of the TA29 promoter, with and without the plastid targeting by the Rubisco small subunit transit peptide. After initial selection on kanamycin, plants transformed with these genes were screened for tissue-specific expression by comparing $P450_{SU1}$ mRNA levels in leaves and anthers on northern blots (data not shown). Previous analyses of many tobacco transformants bearing the TA29 promoter regulating expression of the β-glucuronidase marker gene showed that the TA29 promoter does not always maintain its tissue specificity when it is part of a transgene (J.T. Odell, unpublished results), possibly due to effects of the adjacent genomic DNA. Plants identified as having greater expression of $P450_{SU1}$ in the anthers than in the leaves were then tested for their response to R7402.

Flowering plants were sprayed with R7402 after removing buds that had progressed to a stage in development in which pollen grains are forming and the tapetum tissue is naturally degenerating (Koltunow et al., 1990). After applying R7402 to both wild-type control plants and transformants at rates between 74 μg and 2 mg per plant, an application of 592 μg per plant was determined to give the best effect on transformed pollen without affecting the whole plant or wild-type pollen. Pollen from newly opened buds was collected between 1 and 3 weeks after spraying and tested for the ability to germinate in vitro. Of six different transformants tested where the $P450_{SU1}$ was not targeted to the plastid, there was

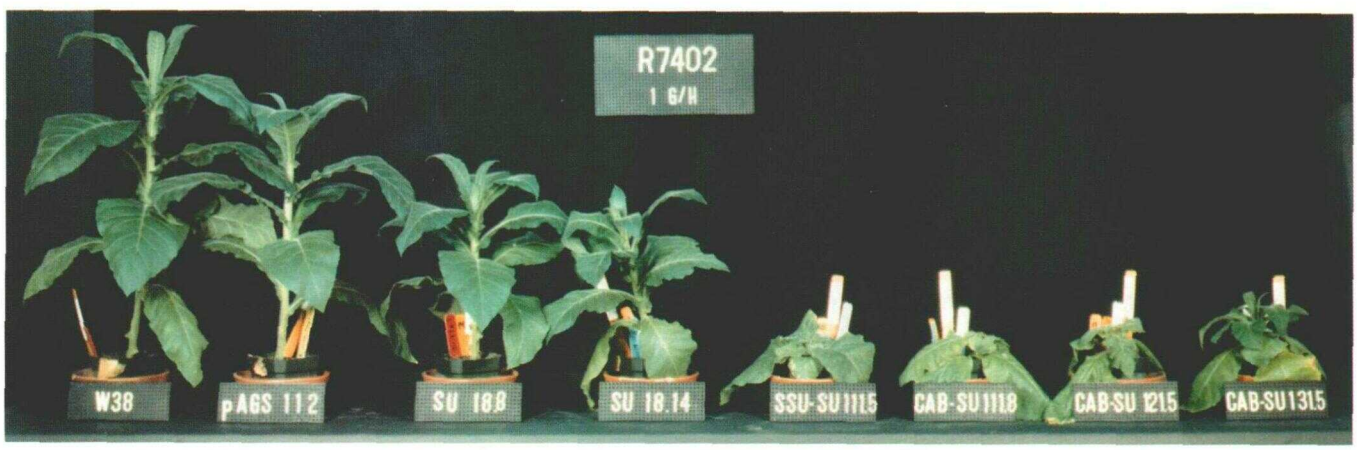

Figure 5. Tobacco plants treated with R7402. Individual plants shown, containing the indicated chimeric genes, had been sprayed 22 d previously with R7402. W38 and pAGS112 are controls, SU18.8 and SU18.14 have cytoplasmic $P450_{SU1}$, and the remainder (SSU-SU111.5, Cab-SU111.8, Cab-SU121.5, and Cab-SU131.5) have chloroplast-directed $P450_{SU1}$.

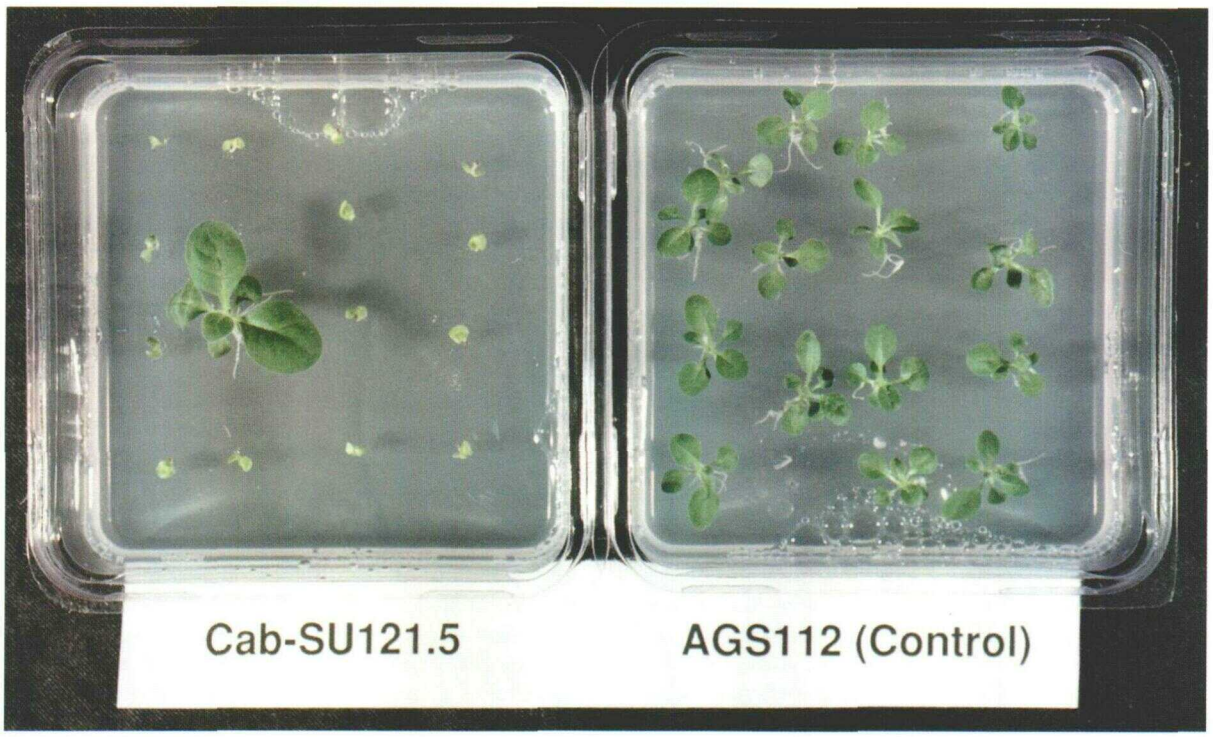

Figure 6. Negative selection of transgenic tobacco. Pictured are plants 21 d after seeds were placed on medium containing 50 nM R7402.

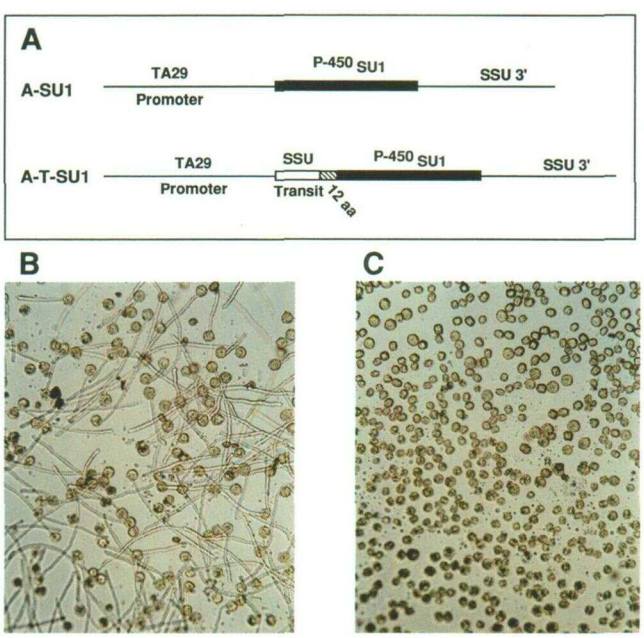

Figure 7. Anther-specific expression of $P450_{SU1}$. A, The chimeric gene constructions using a tapetum-specific promoter to control expression of the P450, with and without the SSU transit sequence. Pollen was obtained from the flowers of plants 7 d after treatment with R7402 and placed on germination media to observe the growth of the pollen tube. B, Pollen from control plant. C, Pollen from a plant with anther-specific expression of SSU transit + $P450_{SU1}$.

little to no effect of R7402 application on pollen viability. Of the 10 plants assayed that had a plastid-targeted $P450_{SU1}$, 7 showed dramatic pollen sensitivity to R7402. Although pollen from control plants taken from newly opened flowers at 7 d after treatment germinated at a rate of 90 to 95%, pollen from the transformants was unable to germinate (Fig. 7, B and C). Pollen samples taken from different transformants at 11, 14, and 21 d germinated to varying degrees. Germination of pollen from the most responsive plant remained at 0 at 11 d, was less than 1% at 18 d, and recovered to 80% at 21 d. The response in another plant was similar, whereas others recovered more quickly. Pollen from all plants expressing $P450_{SU1}$ germinated normally in the absence of R7402 treatment.

DISCUSSION

The results presented here demonstrate that functional plant expression of bacterial $P450_{SU1}$ requires that it be targeted to the chloroplast stromal space. Expression has been demonstrated by the presence of both mRNA and antigen; function has been demonstrated as sensitivity to the compound R7402, which is metabolized to a more phytotoxic form by the monooxygenase activity of $P450_{SU1}$. Although expression without chloroplast targeting results in detectable accumulation of protein, R7402 sensitivity is barely detectable. These results are consistent with the interpretation that the P450 needs to interact with Fd and/or other redox donors

in the chloroplast stromal space to carry out its monooxygenase function. Endogenous plant P450s and NADPH:P450 reductases are generally localized in the ER (functionally isolated as the major component of the microsomal fraction). Extensive studies in other eukaryotes have shown that the NADPH:P450 reductase is located on the cytoplasmic face of the ER membrane and therefore should be accessible to a cytoplasmic protein. When a rabbit liver P450 (CYP2C14) was expressed in tobacco, the protein was found in the microsomal membrane, and the resulting phenotype suggested that the P450 was functional and able to interact with the endogenous plant reductase components (Saito et al., 1991). Although NADPH:P450 reductase should be accessible to the cytoplasmic soluble $P450_{SU1}$, the lack of activity suggests that the eukaryotic-type microsomal P450 reductase is not an efficient electron donor, perhaps because $P450_{SU1}$ has a strict requirement for an Fd reductant.

Although these experiments demonstrate that the P450 in the chloroplast is functional, it is not clear how well it is working. It is possible to obtain crude estimates of typical photosynthetic yields from leaf tissue such as that used in Figure 4 (Bassham, 1977; Walker, 1980). The rate of R7402 N-dealkylation in Cab-SU131.5 corresponds to an efficiency of only 0.0005% for the $P450_{SU1}$ use of photosynthetic reducing equivalents. The extremely small diversion of reducing equivalents to P450 suggests that there is negligible interference with normal metabolism and that only a small amount of P450 is functioning. In support of this interpretation, efforts to measure native P450 spectroscopically in chloroplast stromal contents from SSU-SU121.3 plants were unsuccessful despite levels of antigen demonstrating that detectable quantities of the protein were present. These results suggest that the expression efficiency of native P450 in the chloroplast is far from optimal. Steps such as heme insertion or protein folding may not function optimally for this bacterial protein; consequently, the chloroplast may contain significant levels of inactive protein. In this context, it is notable that despite barely detectable levels of antigen in any of the Cab-SU121.5 plants (Fig. 2), they have levels of sensitivity to R7402 (I_{50} approximately 5 nM when assayed as in Fig. 6) indistinguishable from those of plants with much higher levels of antigen (SSU-SU111.5, SSU-SU121.3, Cab-SU111.8, and Cab-Su131.5). Comparison of these antigen and activity levels also does not provide any compelling information regarding the value of the extra N-terminal amino acids in the processing of the foreign protein.

$P450_{SU1}$ expression is easily measured by R7402 sensitivity in both enzymic and phenotypic assays in transgenic plants, but it provides only marginal resistance to normally phytotoxic herbicides (data not shown). Previous reports have shown that the metabolism in *S. griseolus* that produces metabolites with lowered plant toxicity is primarily carried out by the other inducible enzyme, $P450_{SU2}$ (O'Keefe et al., 1993). Plants expressing this P450, using the strategies described here, would be expected to have significant resistance to some normally phytotoxic herbicides. Other bacterial enzymes have been transformed into plants to increase their herbicide detoxification capabilities, although none have used P450s. These include a phosphonothricin acetyltranferase (DeBlock et al., 1987), bromoxynil nitrilase (Stalker et al., 1988), and a 2,4-D-dealkylating enzyme that is "monooxygenase-like," but not a P450 (Streber and Willmitzer, 1989).

Aside from the demonstration that bacterial P450s can be made to function in higher plants, the availability of a gene whose product activates a pro-herbicide suggests several practical possibilities. Figure 6 demonstrated one of these, which is the use as a negatively selectable marker gene. Such a marker could be a useful tool in gene-targeting studies as a means of selecting out random integration transformants in favor of the low-frequency homologous recombinants. Negative selection has been crucial for the development of targeted gene replacement in mammalian cells (Capecchi, 1989). Other negative selection systems being developed for use in plants include an antisense approach using the *nptII* gene (Xiang and Guerra, 1993) and conversion of 5-fluorocytosine to cytotoxic 5-fluorouracil by bacterial cytosine deaminase (Perara et al., 1993; Stougaard, 1993). Negative selection systems may also be valuable in studies aimed at identifying factors involved in gene regulation. A strategy designed to obtain mutants in the phytochrome signal-transduction pathway combined a *cab140* promoter and the *iaaH* coding region (Brussian et al., 1993). Indoleacetamide hydrolase, encoded by the *iaaH* gene of *A. tumefaciens*, converts naphthalene acetamide to naphthalene acetic acid, which is toxic at high levels (Depicker et al., 1988). The *iaaH* gene was also used as a negative selection in a scheme designed to trap a transposable element in petunia (Renckens et al., 1992). Whether the R7402/$P450_{SU1}$ system will be more effective than these other systems currently in use or being developed is yet to be determined.

Another utility for this gene is as a means of damaging selected plant tissue using the lethal effects of the herbicide as shown in Figure 7. By specifically expressing P450 in the tapetum of the anther in the tobacco flower, it is possible to use R7402 to make the pollen in these flowers nonviable. The sensitivity of pollen development to perturbations in the tapetum has been shown previously. Male sterility has been created genetically by expressing in the tapetum a toxic ribonuclease gene (Mariani et al., 1990) or an antisense RNA to chalcone synthase (van der Meer et al., 1992). The advantage of the R7402/$P450_{SU1}$ system is that the option of chemical application allows choice between viable and nonviable pollen. This may provide a strategy for development of a chemical male sterilant for hybrid seed production.

Other tissue-specific promoters would be expected to yield localized tissue damage similar to that demonstrated in tobacco anthers. It is probably necessary that the selected tissue have some photosynthetic capacity and be physically isolated enough that the R7402 metabolite does not damage surrounding tissue.

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